Kinetics and Regulation of Two Catalytic Subunits of cAMP-Dependent Protein Kinase from *Aplysia californica*†

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ABSTRACT: C_{APL-A1} and C_{APL-A2}, two catalytic subunits of *Aplysia* cAMP-dependent protein kinase, are encoded by mRNAs generated by alternative splicing of transcripts of a gene that contains two mutually exclusive exon cassettes. The subunits are identical except for amino acids 142–183 of the 352 residues, which differ at 10 of 42 positions. C_{APL-A1} and C_{APL-A2} have now been expressed in insect cells and purified to homogeneity. The subunits differ in their catalytic properties, which have been determined with a series of synthetic peptide substrates. For example, k_{cat} and K_m values for the peptide LRRASLG (kemptide) are 42 s^{-1} and 36 μM and 28 s^{-1} and 17 μM for C_{APL-A1} and C_{APL-A2}, respectively. C_{APL-A1} and C_{APL-A2} have different substrate specificities. For example, $(k_{cat}/K_m)_{peptide-T}/(k_{cat}/K_m)_{kemptide}$ is 9.1×10^{-3} for C_{APL-A1} and 15×10^{-3} for C_{APL-A2}, where peptide-T is the kemptide homologue LRRATLG. The subunits also differ in regulation as determined by their interactions with a purified type I regulatory subunit, which has an IC_{50} for C_{APL-A1} that is 3.5 times higher than the IC_{50} for C_{APL-A2}. These modest differences reinforce accumulating evidence that the physiological state of a cell depends upon a spectrum of protein kinases with overlapping substrate specificities and regulatory properties.

A variety of neurons are able to alter their properties in response to external stimuli. This phenomenon, which underlies physiological processes including learning and memory, is known as neuronal modulation (Kaczmarek & Levitan, 1987). One molecular mechanism for neuronal modulation involves the release of neuromodulatory transmitters, which activate signal transduction pathways in target neurons (Kandel & Schwartz, 1982; Nicoll et al., 1988; Kennedy, 1989; Huganir & Greengard, 1990). In *Aplysia californica*, which has a simple nervous system amenable to both electrophysiological and biochemical studies, cAMP-dependent protein kinase (PKA)[1] takes part in neuronal modulation at several sites, including sensory cells at sensorimotor synapses (Kandel & Schwartz, 1982), the bursting neuron R15 of the abdominal ganglion (Levitan et al., 1987), the neuropeptide-secreting bag cells (Kauer & Kaczmarek, 1985), and the circadian pacemaker neurons of the eye (Jacklet, 1989). The properties of muscle cells in the buccal mass (Lloyd et al., 1984) and perhaps the heart (Koester & Koch, 1987) are also regulated by signal transduction pathways involving cAMP.

To understand neuronal modulation and related phenomena in *Aplysia* more fully, the diverse forms of PKA in this species must be defined and the properties of the various subunits determined. To this end, we earlier analyzed cDNAs encoding some of the neuronal catalytic (C) and regulatory (R) subunits of the enzyme (Beushausen et al., 1988; Bergold et al., 1991). In *Aplysia*, the C subunits of PKA are encoded by alternatively spliced RNAs that are transcribed from a single gene, C_{APL-A}, and contain sequences derived from one or the other of two exon cassettes, A1 and A2 (Beushausen et al., 1988), corresponding to exon 6 of the mouse (Chrivia et al., 1988). The peptide sequences encoded by the two exons differ at 10 out of 42 amino acids and lie near the midpoints of the polypeptide chains (residues 142–183 out of 352). Because these sequences encompass and flank residues that are of importance in other species for catalytic activity and modulation by the R subunit (Cameron et al., 1988; Levin et al., 1988; Levin & Zoller, 1990; Taylor et al., 1990; Figure 1) and because they result from a relatively ancient exon duplication (Beushausen et al., 1988), we decided to examine the properties of the two *Aplysia* forms, known as C_{APL-A1} and C_{APL-A2}, to ascertain the extent to which they differ. Because the C subunits are present at low concentrations in *Aplysia* tissues and are not readily separated, we used heterologous expression in insect cells to obtain homogeneous C_{APL-A1} and C_{APL-A2} in sufficient quantities for analysis.

We find that C_{APL-A1} and C_{APL-A2} have similar but discernably different interactions with both synthetic peptide substrates and a type I R subunit. The catalytic domains of the closely related kinases PKA, PKG, and PKC (the AGC kinases) are already known to phosphorylate multiple substrates with overlapping specificities. The finding that isoforms of "individual" kinases, such as PKA (this work) and PKC (Marais & Parker, 1990), have divergent but overlapping properties adds credence to the idea that kinases of the AGC subfamily possess a spectrum of properties that are required for establishing the physiological state of a cell.

Materials and Methods

Materials. Reagents for routine molecular biology were obtained from New England Biolabs and Bethesda Research Laboratories. Plasmids were propagated in *Escherichia coli* XL-1 (Stratagene) unless otherwise stated. Chemicals and

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[1] Abbreviations: AcNPV, the baculovirus *Autographa californica* nuclear polyhedrosis virus; AGC kinase, a member of the protein kinase subfamily that includes PKA, PKG, and PKC; βME, 2-mercaptoethanol; BSA, bovine serum albumin; C subunit, catalytic subunit of PKA; C_{APL-A1}, isoform of the C subunit of *Aplysia* PKA including the sequence encoded by alternative exon A1; C_{APL-A2}, isoform of the C subunit of *Aplysia* PKA including the sequence encoded by alternative exon A2; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; IBMX, 1-isobutylmethylxanthine; MES, 4-morpholineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NP-40, Nonidet P-40; pfu, plaque-forming unit; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PKIP, protein kinase inhibitor peptide; PMSF, phenylmethanesulfonyl fluoride; R subunit, regulatory subunit of PKA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SFM, serum-free medium; UTR, untranslated region.

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A2                      | S         I SF    Y  LV            PQ  C |
A1      GGEMFSHLRRIGRFS |EPHSRFYAAQIVLVLEYLHHLDIMYRDLKPENLLIDSYGYLK| VTDFGFAKRVKGRTWTLCGTPEYLAPE
                        |                         •••  #• #        |   •••          # #      •••
                        |                                          |   #
DC0              KV     | P         V AF    Y  LI            SQ  L |
Mα                      | P A       V TF    S  LI            QQ  IQ|
Mβ                      | P A       V TF    S  LI            HQ  IQ|
Hγ            R Q V     | P AC     VV AVQ   S  LIH           QQ  LQ|
TPK1       L  L  KSQ  P |NPVAK    EVC AL    SK II        I L KN HI | I       Y PDV Y      D I
TPK2       L  L  KSQ  P |NPVAK    EVI AL    AHNII        I L RN HI | I       E QT         D I
TPK3       L  L  KSQ  P |NPVAK    EVC AL    SK IT        I L KN HI | I       Y PDV Y      D I
B-PKG      LWTI  DR S E |DSTT   T CVVEAFA   SKGII         IL HR  A | LV      KIG3K   F      V
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% identity to alternative sequence of:

	A1	A2
A1	100	76
A2	76	100
DC0	86	86
Mα	76	79
Mβ	76	79
Hγ	69	67
TPK1	60	52
TPK2	57	52
TPK3	60	52
B-PKG	50	50

FIGURE 1: Alignment of the amino acid sequences of several kinase catalytic domains in the vicinity of the sequences encoded by exons A1 and A2 in *Aplysia*. The entire sequence from residue 127 to residue 210 is shown for C_{APL-A1} (A1). The numbering is according to Beushausen et al. (1988). All the variant amino acids in C_{APL-A2} are shown above (A2). The amino acids at variant positions are indicated for the other sequences whether they differ from C_{APL-A1}, from C_{APL-A2}, or from both [DC0, a *Drosophila* PKA C subunit (Foster et al., 1988; Kalderon & Rubin, 1988); Mα, mouse PKA Cα (Chrivia et al., 1988); Mβ, mouse PKA Cβ (Chrivia et al., 1988); Hγ, human PKA Cγ (Beebe et al., 1990); TPK1, TPK2, and TPK3, *S. cerevisiae* homologues of PKA C subunits (Toda et al., 1987); B-PKG, bovine PKG Iα catalytic domain (Takio et al., 1984)]. The region encoded by the alternative exon in *Aplysia* is boxed. A corresponding exon exists in the mouse [exon 6 (Chrivia et al., 1988)], probably as a single functional copy (Beushausen et al., 1988). The *Drosophila* and yeast genes have no introns in the coding region. The structures of the Cγ and PKG Iα genes have not been determined. Residues that are important for catalytic activity or interaction with the R subunit as determined by site-directed mutagenesis or chemical modification of mammalian and yeast subunits are marked (#) (Cameron et al., 1988; Levin et al., 1988; Levin & Zoller, 1990; Taylor et al., 1990). The number "3" in the PKG Iα sequence indicates an insertion of two residues. Sequences highly conserved in protein kinases are marked (●) (Hanks et al., 1988). The percent identity to the sequences encoded by A1 and A2 are shown beneath the alignment.

buffer components including protease inhibitors were from Sigma unless otherwise noted. Nitrocellulose was purchased from Schleicher & Schuell. Grace's insect cell culture medium powder was from Hazleton and serum-free liquid medium (Sf900) from Gibco. Yeastolate and lactalbumin hydrolysate were obtained from Difco. Colloidal gold protein stain (AuroDye forte) was from Amersham Corporation. Bradford protein assay reagent, low-melt agarose, and desalting resin (Bio-Gel P-6DG) were supplied by Bio-Rad. [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were obtained from New England Nuclear. (Diethylamino)ethyl cellulose (DE-52) and phosphocellulose (P11) ion exchange resins and P81 phosphocellulose paper were supplied by Whatman Biosystems Ltd. Peptide substrates were from Bachem except for kemptide (LRRASLG) and peptide-G (RKRSRKE), which were from Peninsula, and peptide-T (LRRATLG) and truncated protein kinase inhibitor peptide (PKIP 18-mer; sequence: TTYADFIASGRTGRRNAI), which were synthesized by John Leszyk at the Protein Chemistry Facility (Worcester Foundation). For affinity chromatography, crude PKIP was transferred to 0.1 M MOPS (pH 8.5) by gel filtration through Bio-Gel P-6DG and coupled to Affi-Gel 10 at 0.32 μmol/mL as described by Olsen and Uhler (1989). Oligonucleotides were made at the Cancer Center Core Facility at the Worcester Foundation.

Construction of a cDNA Containing the Entire Coding Region of C_{APL-A2}. The cDNA, AC-A2, encoding C_{APL-A2}, previously isolated from a neuronal cDNA library (Beushausen et al., 1988), had an apparently aberrant 5′ end, which was replaced by the 5′ end of cDNA AC-1b (Beushausen et al., 1988) by ligating the following DNAs in a single reaction: (i) the 5′ region of AC-1b from the *Xba*I site in the phage linker to the *Eco*RI site at position 52 in the cDNA; (ii) the 3′ region of AC-A2 from the *Eco*RI site to a *Sac*I site in the phage linker; (iii) *Xba*I–*Sac*I cut Bluescript KS(−). The resulting plasmid, pKS-C_{APL-A2} (S. Beushausen, unpublished work), contains an insert with 168 bp of 5′ UTR and 40 bp of 3′ UTR. We have recently shown that the 5′ end of AC-A2 arises from an exon encoding an alternative N-terminus (N2) and that there are at least four transcripts encoding the C subunit of PKA in *Aplysia* (S. Beushausen, E. Lee, B. Walker, and H. Bayley, submitted for publication). mRNAs for the two forms of C_{APL-A} protein purified in the present work are abundant transcripts in *Aplysia* and encode the N-terminus (N1) described previously (Beushausen et al., 1988), which is homologous to the mammalian form.

Introduction of an NdeI Site at the Initiator Met of C_{APL-A2}. We first attempted to express C_{APL-A2} in *E. coli* using a pT7 vector requiring the cDNA to be inserted at an *Nde*I site (CA′TATG) the appropriate distance from a Shine–Delgarno sequence (S. Cheley, D. Milkes, and H. Bayley, unpublished work). The new restriction site was introduced into the cDNA by site-directed mutagenesis. In brief, single-stranded pKS-C_{APL-A2} was obtained by rescue with M13KO7 helper phage and hybridized to the mutagenic oligonucleotide pTTACACTCAGcATATGGGCAATG. The underlined ATG is the initiator Met and the mismatch is in lower case. Second-strand synthesis was completed using the dual primer procedure of Zoller and Smith (1987). Mutated plasmids were selected by digestion with *Nde*I and hybridization to ^{32}P-labeled CACTCAGCATATGGGCA. After retransformation

of *E. coli* to ensure the isolation of clones, plasmid pKS-C_{APL-A2}-11A was used for further work.

Insertion of C_{APL-A2} cDNA into the Expression Vector pT7-f1A. The modified C_{APL-A2} cDNA was removed from Bluescript KS(−) with *Sac*I endonuclease, utilizing a site in the 5′ linker immediately 3′ of the *Xba*I site and the *Sac*I site in the 3′ linker. The *Sac*I 3′ overhangs were made blunt by mild S1 nuclease treatment and repaired using Klenow enzyme. *Hin*dIII linkers were then ligated onto the blunt ends. Both the cDNA and the pT7-f1A vector (Kuret et al., 1988) were digested to completion with *Nde*I and *Hin*dIII. Gel-purified cDNA and vector were ligated, producing pT7-C_{APL-A2}, a plasmid with C_{APL-A2} DNA properly positioned and oriented in the vector for expression from the T7 promoter.

Construction of the pT7 Expression Vector Containing C_{APL-A1}. A central section of the C_{APL-A2} cDNA in pT7-C_{APL-A2} was removed by digestion with *Ava*I (position 625) and partial cleavage with *Eco*RI (position 52; there is also a site in the vector). This region included exon A2, which corresponds to exon 6 of the mouse, the only region of diversity between the two forms of the *Aplysia* catalytic subunit described here. The homologous region of C_{APL-A1} cDNA was removed from plasmid pKS-C_{APL-A1} with the same endonucleases and used to replace the excised region of pT7-C_{APL-A2} yielding pT7-C_{APL-A1}. pKS-C_{APL-A1} is a similar construct to pKS-C_{APL-A2} containing the entire coding region of C_{APL-A1}. The plasmid was constructed using the *Xba*I–*Bst*EII fragment of cDNA AC-A1b and the *Bst*EII–*Sac*I fragment of AC-A1a (S. Beushausen, unpublished work). The *Bst*EII site is at position 538 in exon A1. The *Sac*I site in AC-A1a is in the phage linker leaving 600 bp of 3′ UTR.

Sequence Verification. The sequences of the coding regions of pT7-C_{APL-A1} and pT7-C_{APL-A2} were determined by dideoxy chain termination using modified T7 DNA polymerase (Sequenase, USB) and a series of seven primers. The sequences were identical to those reported by Beushausen et al. (1988) except that position 1050 was C rather than T in both constructs. Codon 350 becomes GCC, instead of GCT, but still codes for Ala.

Transfer Vectors pVL-C_{APL-A1} and pVL-C_{APL-A2}. Transfer vector pVL941 was provided by Drs. V. Luckow and M. Summers (Luckow & Summers, 1989). Recombinant transfer vectors pVL-C_{APL-A1} and pVL-C_{APL-A2} were constructed as follows. cDNA fragments (1.1 kb) encoding C_{APL-A1} or C_{APL-A2} were excised from *E. coli* expression vectors pT7-C_{APL-A1} and pT7-C_{APL-A2} with *Hin*dIII and *Nde*I. After fill in with Klenow enzyme, these fragments were ligated to *Bam*HI linkers and inserted at the unique *Bam*HI site of pVL941 downstream from the polyhedrin promoter. The orientations of the C_{APL-A} inserts were determined by digestion with *Eco*RV (Figure 2).

Cell and Virus Culture. Spodoptera frugiperda (Sf9) cells were obtained from the American Type Culture Collection and were maintained at 28 °C in TNM-FH medium (Hink, 1970) supplemented with 5% fetal calf serum (FCS) and 50 μg/mL gentamycin ("normal medium"). Baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) was provided by Dr. M. Summers. Sf9 cells were infected with wild-type or recombinant virus at a multiplicity of 0.1–1 plaque forming units (pfu) for virus amplification and 5–10 pfu for expression. For expression, TNM-FH medium was replaced 40 h post-infection with serum-free medium (Sf900 medium, Gibco) supplemented with 0.5% FCS.

Production of Recombinant Baculovirus. Recombinant *Baculovirus* was produced by cotransfection of Sf9 cells (2

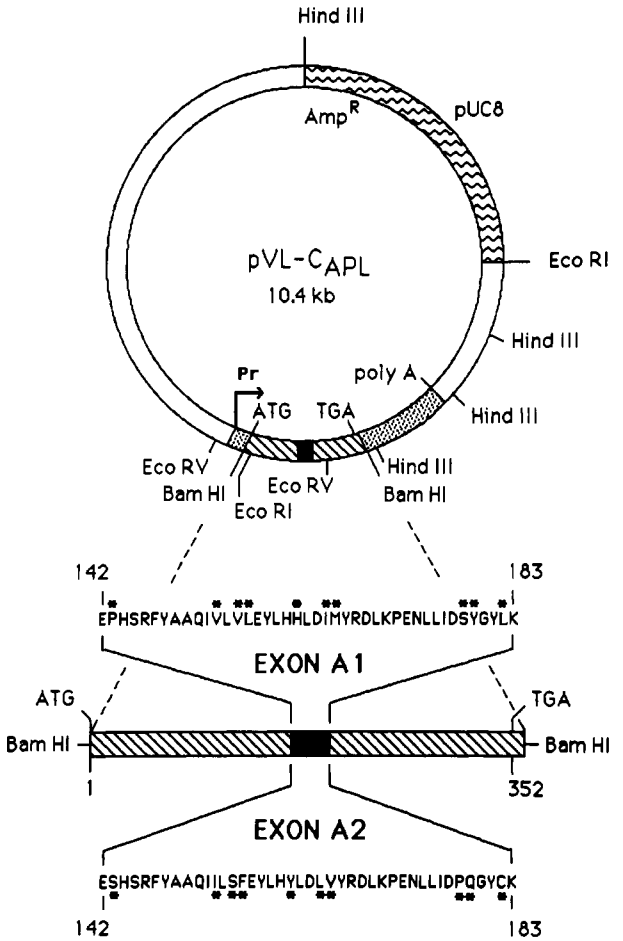

FIGURE 2: Transfer vectors pVL-C_{APL-A1} and pVL-C_{APL-A2}. cDNAs encoding *Aplysia* C subunit isoforms C_{APL-A1} and C_{APL-A2} were inserted at the unique *Bam*H1 site of pVL941. The solid area demarks the internal isoform-specific region generated by the mutually exclusive splicing of exon cassettes (Beushausen et al., 1988). The amino acids encoded by these cassettes (A1 and A2) are shown and substitutions are marked (*). Kinase sequences common to both isoforms are shaded. Stippled flanking sequences contain the polyhedrin gene promoter region (Pr), untranslated sequences and a polyadenylation signal.

× 10^6 cells) with 1 μg of AcNPV DNA and 2 μg of either CsCl gradient-purified pVL-C_{APL-A1} or pVL-C_{APL-A2}. Recombinant virus derived from the supernatant medium of transfection cultures was purified by three rounds of limiting dilution in 96-well microtiter plates (Fung et al., 1988). For each round of purification, levels of expressed C_{APL-A1} and C_{APL-A2} in cell lysates were analyzed by dot assays for kinase activity and by dot-blot hybridization. For dot assays, supernatant medium was removed from the multiwell dishes and stored at 4 °C. Cells were lysed in the multiwell dishes with 50 μL of lysis buffer (10 mM Tris·HCl, pH 7.5, 1.0% NP-40, 10% glycerol) and one freeze–thaw cycle. Aliquots of these multiwell lysates (5 μL) were transferred to another 96-well plate and mixed with 45 μL of kinase assay mix containing the synthetic substrate kemptide (see assay conditions below). After 20 min at room temperature, 40 μL of each multiwell assay mix was applied to the wells of a filtration manifold containing P-81 paper that had been overlayed with 100 μL of 75 mM phosphoric acid per well. After filtration, 75 mM phosphoric acid was washed through the wells. After removal from the manifold, the sheets were further washed with 75 mM phosphoric acid and exposed to X-ray film for 10 min. For dot-blot hybridization, 20 μL of the above lysates were mixed with 20 μL of 0.4 M NaOH and applied to the wells of a

Table I: Typical Purification of *Aplysia* C_{APL-A1} from Insect Cells

step	mass of protein (μg)	tot act. (μmol/min)	spec act. [μmol/(min·mg)][a]	fold purification	yield (%)
Sf9 supernatant	150000	5.2	0.033	1	100
DEAE-phosphocellulose tandem column	1400	2.5	1.8	55	48
PKIP-Affi-Gel	29	1.6	55	1700	31

[a] Determined using 100 μM kemptide. k_{cat} values for C_{APL-A1} and C_{APL-A2} are given in Table II.

filtration manifold containing nitrocellulose paper that had been overlayed with 100 μL of 2 M NaCl per well. After filtration, blots were baked for 2 h at 80 °C under vacuum and probed with a ^{32}P-labeled sense-strand RNA derived by in vitro transcription of linearized pT7-C_{APL-A1} or pT7-C_{APL-A2} DNA with T7 RNA polymerase in the presence of [α-^{32}P]-UTP.

Production and Purification of C_{APL-A1} and C_{APL-A2}. Two large monolayer flasks (24.5 × 24.5 cm; Nunc) were seeded with Sf9 cells derived from spinner flasks and grown in TNM-FH, containing 5% FCS. At 75% confluency, the medium was removed, and the monolayers were allowed to adsorb recombinant AcNPV-C_{APL-A1} or AcNPV-C_{APL-A2} virus at a multiplicity of 5–10 pfu/cell for 1 h at room temperature with rocking. Infected cultures were then reincubated at 28 °C in fresh medium. At 40 h postinfection, the medium was replaced with Sf900 medium supplemented with 0.5% FCS. At 80–88 h postinfection, the medium (now containing recombinant C subunit) was harvested, cleared of cellular debris by centrifugation (2000*g*), made 10% (v/v) in glycerol and stored at −80 °C. The medium was replenished with Sf900, containing 0.5% FCS. Two further supernatants were collected after ~20-h intervals. Thereafter, little further kinase was released as determined by assay of the supernatant medium.

For each expression culture, supernatant media from the three sequential harvests were pooled (600-mL total) and passed through a DE-52 column (100-mL bed volume) hooked in tandem to a phosphocellulose P-11 column (50-mL bed volume). The tandem columns were washed with 200 mL of P-EDTA buffer (10 mM sodium phosphate, 1 mM EDTA, 5 mM βMe, pH 7.0) and then disconnected. C_{APL-A1} (or C_{APL-A2}) was eluted from the phosphocellulose column with a gradient of 50–400 mM sodium phosphate in P-EDTA in 50 mM steps. The peak of kinase activity was eluted at 200 and 250 mM phosphate. Subsequent affinity purification of C_{APL-A1} (or C_{APL-A2}) was as described (Olsen & Uhler, 1989) with the following modifications. The PKIP affinity matrix was washed and equilibrated with 1× PKIP binding buffer (20 mM Tris·HCl, 2 mM $MgCl_2$, 1 mM ATP, 0.5 mM PMSF, 1 mM EDTA, 1 mM DTT, and 0.1% NP-40, pH 7.4). Peak phosphocellulose fractions were pooled, mixed 1:1 with 2× PKIP binding buffer and 1 mL (settled volume) of PKIP affinity matrix. The mixture was rotated overnight at 4 °C. The PKIP column was then washed with five bed volumes of 1× binding buffer and five bed volumes of PKIP wash buffer (20 mM Tris·HCl, 0.5 mM PMSF, 0.1% NP-40, 200 mM NaCl). C_{APL-A1} (or C_{APL-A2}) was eluted from the PKIP column with 20 bed volumes (4 × 5 mL) PKIP elution buffer (20 mM Tris·HCl, 200 mM arginine·HCl, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, pH 7.4). To remove arginine, fractions containing peak activity were pooled, and portions (2.5 mL) were centrifuged through a 25-mL Bio-Gel P-6DG desalting spin column equilibrated with buffer containing 20 mM Tris·HCl (pH 7.4), 1 mM DTT, and 1 mM EDTA. The purified C subunits were made 10% (v/v) in glycerol and stored in aliquots at −80 °C. The addition of glycerol was crucial; more than 95% of the activity was lost when the subunits were directly frozen. The results of a typical purification are compiled in Table I.

Arginine inhibits the *Aplysia* C subunits with K_i values of ~60 mM, and it was essential that the concentration be reduced below 1 mM in the kinase assays. Therefore, arginine levels in the purified subunits preparations were checked by amino acid analysis. In the assays described below, the levels found cannot have caused more than 3% inhibition at the lowest substrate concentrations as determined by the expression $V_i/V_m = (1 + [I]/K_i)^{-1}$, derived from the Michaelis–Menton equation, where V_i/V_m is the value to which the ratio of the rates in the presence (V_i) and absence (V_m) of a competitive inhibitor tends at low substrate concentration. [I] is the inhibitor concentration and K_i its dissociation constant. In the assays described below, a linear dependence of rate on enzyme concentration was found, both for C_{APL-A1} and C_{APL-A2}, further ruling out the possibility of contaminating inhibitors.

Protein Kinase Assay. For monitoring protein purification, C_{APL-A1} and C_{APL-A2} were assayed at 30 °C using kemptide (LRRASLG) as a substrate. The reaction mixture contained 50 mM MOPS, pH 6.8, 15 mM $MgCl_2$, 2.5 mM IBMX, 100 μg/mL BSA, 0.1% NP-40, 100 μM kemptide, C subunit, and 100 μM ATP (ultrapure; Pharmacia) containing [γ-^{32}P]ATP at ~550 dpm/pmol in a final volume of 50 μL. Peptide aliquots and reaction mixes were preequilibrated at 30 °C, mixed, and incubated at the same temperature for 20 min. A portion of the mix (40 μL) was then applied to a small square of P81 phosphocellulose paper (Roskowski, 1983). The paper was thoroughly washed in 75 mM phosphoric acid and counted by liquid scintillation spectrometry in Econolume (5 mL; ICN Schwarz-Mann).

Kinetic Parameters for Kemptide. The kinetic parameters for kemptide were determined for the purified C subunits using a similar assay but omitting the phosphodiesterase inhibitor IBMX and using a range of peptide concentrations (6.25–200 μM) and a defined enzyme concentration (20 ng/mL). Protein concentration was determined by the colloidal gold assay described by Cheley and Bayley (1991), kemptide concentration by amino acid analysis, and cold ATP concentration by absorbance, using ϵ_{260} = 15 400. The specific activity of the ATP was determined by scintillation counting. In each experiment assays were carried out in duplicate. A small background correction was made for radioactivity retained by the P81 paper in the absence of enzyme. V_{max} and K_m were determined by fitting the data to the Michaelis–Menton equation using software provided in Sigmaplot (version 4.02, Jandel Scientific), and k_{cat} was calculated using the known protein concentration.

Kinetic Parameters for Other Peptides. For comparative kinetic analyses, the peptide substrates LRRATLG (kemptide-T), RKRSRKE (peptide-G, a substrate for PKG), RRKASGP (peptide-H, a histone fragment), RFARKGSLRQKNV (peptide-C, a substrate for PKC), and RRLSSLRA (peptide-S6, a substrate for S6 kinase) were substituted for kemptide (LRRASLG). The sites of phosphorylation are underlined. For peptide-S6, the site is that phosphorylated by mammalian PKA (Pelech et al., 1986). To

Table II: Kinetic Properties of C_{APL-A1} and C_{APL-A2} Examined with Synthetic Peptide Substrates[a]

peptide	k_{cat}	K_m	k_{cat}/K_m
		C_{APL-A1}	
K	42 ± 4	36.3 ± 0.2	1.2 ± 0.1
T	9.0 ± 1.2	870 ± 130	0.011 ± 0.001
G	2.22 ± 0.03	890 ± 110	0.0025 ± 0.0003
C	49 ± 1	61 ± 11	0.83 ± 0.15
H	35 ± 1	730 ± 50	0.048 ± 0.004
S6	33 ± 1	500 ± 20	0.069 ± 0.003
		C_{APL-A2}	
K	27.5 ± 0.4	17 ± 1	1.6 ± 0.1
T	14 ± 1	590 ± 80	0.025 ± 0.002
G	4.3 ± 0.2	790 ± 80	0.0055 ± 0.0009
C	34 ± 2	27 ± 3	1.3 ± 0.2
H	28 ± 2	354 ± 5	0.080 ± 0.007
S6	27.5 ± 0.1	220 ± 30	0.14 ± 0.01

[a] The peptides are K, LRRASLG; T, LRRATLG; G, RKRSRKE; C, RFARKGSLRQKNV; H, RRKASGP; S6, RRLSSLRA. k_{cat} is in s^{-1}. K_m is in μM. k_{cat} and K_m were determined for kemptide (K) in three separate experiments, each performed in duplicate. The mean of the three values and the standard deviation (σ_{n-1}) are given. k_{cat} and K_m for the other peptides were also determined in three separate experiments, each performed in duplicate. In these experiments the activity of the enzyme preparation was checked with 200 μM kemptide, and the values obtained were used to make small adjustments to the k_{cat} values. The mean of the three values and the standard deviation were then calculated. k_{cat}/K_m values are also the means of the three separate determinations.

determine k_{cat} and K_m for each substrate, C_{APL-A1} and C_{APL-A2} were assayed over a range of peptide concentrations: 6.25–200 μM for peptide-C and peptide-S6, 62.5–2000 μM for peptide-H and peptide-G, and 62.5–4000 μM for peptide-T. For peptide-G the incubation time was increased to 60 min. For all substrates examined, rates were linear over the assay period. As the differences in kinetic parameters between C_{APL-A1} and C_{APL-A2} are small, kemptide (for which k_{cat} had been carefully determined with freshly purified subunits) was always assayed under V_{max} conditions, with the same assay mix used for the peptides, to ensure that the enzyme preparations had not lost activity. In the experiments compiled in Table II, all peptides were assayed on three separate occasions, each time in duplicate with the same assay mixes.

RII Phosphorylation. RIIα was purified from bovine heart essentially as described (Corbin & Rannels, 1981; Rannels et al., 1983), culminating with urea elution from a cAMP-Sepharose affinity column (C8-linked, Sigma no. A0144) and removal of urea by gel filtration on Sephadex G50m. The subunit was at least 90% pure when analyzed by SDS–PAGE. In vitro phosphorylation, by C_{APL-A1} or C_{APL-A2}, was carried out in a mix consisting of 128 nM RIIα in 50 mM MOPS, 15 mM $MgCl_2$, and 100 μg/mL BSA, pH 6.8, containing 10 μM cAMP. The reaction was initiated by the addition of [γ-^{32}P]ATP and C subunit, which had been preincubated together, to 1 nM and 55 pM, respectively. Aliquots were removed at appropriate time intervals over 10 min and mixed immediately with an equal volume of 2× Laemmli sample buffer to stop the reaction. After SDS–PAGE, dried gels were exposed to X-ray film for 2–6 h. The autoradiograms were scanned using a LKB 2222-020 UltroScan XL laser densitometer.

Inhibition by Recombinant Bovine RIα. Bovine RIα was prepared from extracts of *E. coli* 222 transformed with pLST-18 by modifying a procedure provided by W. Yonemoto and S. Taylor. This strain produces RIα with an N-terminal extension of 10 amino acids, which produces no dramatic alterations in the properties of the subunit (Saraswat et al., 1986). In brief, cells were lysed by sonication. rRIα was precipitated with 45% ammonium sulfate, dialyzed, and subjected to two rounds of chromatography on DE-52 eluted with a NaCl gradient in 10 mM MES, 2 mM EDTA, and 5 mM βME, pH 6.5. rRIα was stored frozen after the addition of glycerol to 5%. Bound cAMP was removed by a modification of the procedure of Builder et al. (1980), which minimizes exposure to urea (Corbin & Rannels, 1981). Solid urea was added to rRIα in DE-52 buffer to a concentration of 8 M. The denatured protein was immediately separated from cAMP by chromatography on Sephadex G50m equilibrated at room temperature with 8 M urea, freshly prepared using 50 mM potassium phosphate, 100 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, and 5 mM βME, pH 6.5. The eluted R subunit was detected using the rapid Bradford protein assay (protein blue; urea green). Pooled fractions were applied without delay to a second G50m column equilibrated in 50 mM potassium phosphate, 100 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, and 5 mM βME, pH 6.5, to remove urea, again at room temperature. The refolded, cAMP-free rRIα was kept on ice and used within a few hours.

Two-fold serial dilutions of cAMP-free rRIα were incubated with C subunit in 45 μL of assay buffer (1.11×) in the absence of substrate for 20 min at 30 °C. Phosphorylation was initiated by adding 1 mM kemptide (5 μL) and incubating for a further 20 min at 30 °C. Final concentrations were 50 mM MOPS, 15 mM $MgCl_2$, 100 μg/mL BSA, pH 6.8, 100 μM [γ-^{32}P]-ATP (550 dpm/pmol), 96 pM C subunit, and 100 μM kemptide. Identical concentrations of C_{APL-A1} and C_{APL-A2} were assayed simultaneously with the same preparation of rRIα.

Miscellaneous. The following factors were used to convert literature values of V_{max} to k_{cat}: PKA C subunit, 1.0 μmol/(min·mg) = 0.68 s^{-1}; PKG holoenzyme, 1.0 μmol/(min·mg) = 1.3 s^{-1}. In the examples quoted from the literature, measurements were made on enzymes purified from tissues and were therefore mixtures of isoforms. Unless otherwise stated, the quoted sequence identities of protein kinases in the kinase domain (Hanks et al., 1988) were calculated using DISTANCES (GCG package, University of Wisconsin, Biotechnology Center).

RESULTS

Heterologous Expression of PKA Subunits C_{APL-A1} and C_{APL-A2}. We first attempted to express the *Aplysia* PKA subunits C_{APL-A1} and C_{APL-A2} in *E. coli*. Complete coding regions were constructed as described under Materials and Methods and inserted behind the T7 polymerase promoter in the vector pT7-f1A (Kuret et al., 1988). The resulting plasmids were used to transform *E. coli* BL21 bearing chromosomal copies of T7 polymerase under control of the *lac* UV promoter (Studier et al., 1990). While several strains expressed C_{APL-A1} or C_{APL-A2} at high levels, the polypeptides were largely insoluble and specific kinase activities very low. Numerous variations of the growth conditions and attempts to renature the proteins failed to yield fully active kinase. We next tried to express C_{APL-A1} and C_{APL-A2} in *Saccharomyces cerevisiae* using vectors carrying the alcohol dehydrogenase promoter (S. Cheley and L. Levin, unpublished work). In this case the extent of expression was not high enough (C_{APL-A1} ~30 μg/L; C_{APL-A2} ~10 μg/L) to make a purification practicable. Therefore, we turned to a *Baculovirus* expression system.

C_{APL-A1} and C_{APL-A2} Expressed in Insect Cells. Although insect cell expression did eventually prove successful, it was by no means straightforward. First, purification of recombinant virus was greatly facilitated, compared with purification

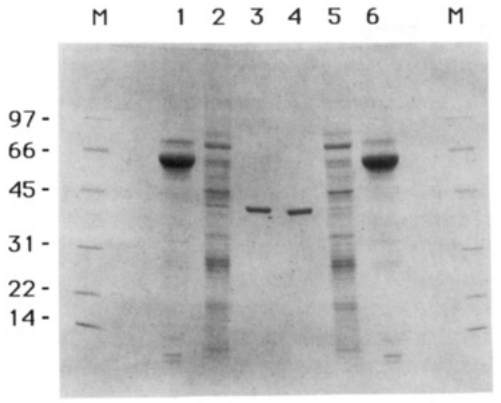

FIGURE 3: Purification of the catalytic subunits C_{APL-A1} and C_{APL-A2} from Sf9 cells infected with recombinant AcNPVs. Protein from various stages of the two purifications were analysed by SDS–PAGE using a 8–18% gradient gel (Laemmli, 1970). (Lanes 1–3) Purification of C_{APL-A1}. (Lanes 4–6) Purification of C_{APL-A2}. Lanes 1 and 6 show supernatant media (25 μg of protein): lanes 2 and 5 show the phosphocellulose fractions containing kinase activity (25 μg of protein); lanes 3 and 4 show the purified C subunits (2.5 μg in each lane). The molecular weight markers (M, Bio-Rad) are phosphorylase b (97 000); bovine serum albumin (66 000); ovalbumin (45 000); carbonic anhydrase (31 000); soy bean trypsin inhibitor (22 000); lysozyme (14 000).

by plaque morphology, by combining the technique of limiting dilution (Fung et al., 1988) with kinase assays of individual wells.

Second, most of the active kinase was found in supernatants rather than the monolayers themselves, presumably due to the progressive lysis of infected cells. Therefore, a balance was sought between higher levels of expression in normal medium [5–10% fetal calf serum (FCS)] and the ease of purification from serum-free medium (SFM). Cells adapted to SFM or unadapted cells grown in SFM throughout an expression culture expressed C_{APL-A1} and C_{APL-A2} poorly (at least 5 times less well than the method subsequently developed). As a compromise, cells in monolayers were infected in normal medium, and 40 h postinfection, when kinase activity appears in the supernatant, the medium was changed to SFM containing 0.5% FCS. Using this maneuver, we obtained expression levels of up to one-half of those in normal medium. Yields were further increased by continuously harvesting the culture supernatant from 80 h to 120 h postinfection.

Despite these efforts, the yields of *Aplysia* C subunits were very low compared with those of many other recombinant proteins in Sf9 cells (Luckow, 1990). Spinner cultures gave yet lower yields. This may in part be due to an interesting change in morphology of Sf9 cells expressing C_{APL-A1} and C_{APL-A2}: the infected cells extend long neurite-like processes (S. Cheley, unpublished observations).

Purification of C_{APL-A1} and C_{APL-A2}. The C subunits were purified from the Sf9 supernatants by a two-step procedure that could be carried out in less than a day (Table I). The second step of affinity chromatography on PKIP-Affi-Gel (Olsen & Uhler, 1989)[2] yielded highly purified C_{APL-A1} or C_{APL-A2} as judged by SDS-PAGE (Figure 3), which also revealed the same slight difference in apparent molecular weight ($\Delta \sim 700$ daltons) noted for the polypeptides expressed in bacteria (data not shown). In vitro translation products of T7 RNA polymerase transcripts also showed the same apparent molecular weight difference. When C_{APL-A1} or C_{APL-A2} was adsorbed to the affinity matrix in low salt, considerable kinase activity was eluted in the salt washes prior to elution with arginine, perhaps as a result of secondary adsorption of the kinase to the protein-saturated column. This was reduced by carrying out the adsorption in the presence of 100–125 mM sodium phosphate buffer. The weak inhibitor arginine (Demaille et al., 1977) was removed from the eluted kinase by gel filtration. The specific activities of purified C_{APL-A1} or C_{APL-A2} were similar to those found for the mammalian C subunit (see below).[3] Any inactive protein was presumably removed by using an affinity column requiring an intact active site in the last step of the purification. The dilute subunits were immediately stored and frozen with 10% glycerol, avoiding further manipulations such as a concentration step.

C_{APL-A1} and C_{APL-A2} Exhibit Different Kinetic Constants for the Synthetic Peptide Substrate Kemptide. Because they form the basis for the comparative values derived for other peptide substrates (see below), considerable care was taken in measuring the kinetic constants k_{cat} and K_m for C_{APL-A1} and C_{APL-A2}. For example, each set of measurements on C_{APL-A1} and C_{APL-A2} was carried out on the same day using the same reaction mixes. Other precautions are described under Materials and Methods.

The k_{cat} values for C_{APL-A1} ($42 \pm 4\ s^{-1}$; Table II) and C_{APL-A2} ($27.5 \pm 0.4\ s^{-1}$) are of the same order of magnitude as those reported for the bovine enzyme (e.g., $14\ s^{-1}$; Kemp et al., 1977). As our protein assays were done using colloidal gold and a BSA standard (Cheley & Bayley, 1991), the k_{cat} values reported here may not be strictly comparable with those obtained previously for C subunits from other species. However, the relative values for C_{APL-A1} and C_{APL-A2}, which are our main concern in this paper, should be valid provided that the two subunits bind gold equally well. The value of k_{cat-A1} for kemptide is ~1.5-fold higher than k_{cat-A2}. In contrast, C_{APL-A1} "bound" kemptide more weakly than C_{APL-A2}, the ratio of K_{m-A1} (36.3 ± 0.2 μM) to K_{m-A2} (17 ± 1.0 μM) being 2.1. In experiments with C_{APL-A1} and C_{APL-A2}, partly purified after expression in *S. cerevisiae*, this ratio was 2.3. The K_m for kemptide of the bovine C subunit is 16 μM (presumably predominantly Cα; Kemp et al., 1977), which therefore resembles C_{APL-A2} more closely than C_{APL-A1} in its interactions with kemptide.

The relative specificity of C_{APL-A1} and C_{APL-A2} for a substrate can be calculated from the relationship

$$\frac{V_{A1}}{V_{A2}} = \frac{(k_{cat-A1}/K_{m-A1})[C_{APL-A1}]}{(k_{cat-A2}/K_{m-A2})[C_{APL-A2}]}$$

Letting the enzyme concentrations be equal, this ratio is 0.71 for kemptide. In other words, if equimolar amounts of C_{APL-A1} and C_{APL-A2} were competing for kemptide, C_{APL-A2} would phosphorylate it 1.4 times more rapidly. Because the differences in k_{cat} and K_m between C_{APL-A1} and C_{APL-A2} oppose each other, the difference in relative specificity is modest though significant (for standard deviations in k_{cat}/K_m values, see Table II).

C_{APL-A1} and C_{APL-A2} Have Different Kinetic Constants for Several Other Synthetic Peptide Substrates. For comparison with kemptide, kinetic constants were determined for five other peptides substrates (Table II): peptide-T [kemptide in which the phosphorylated serine residue is replaced by threonine (Kemp et al., 1977)]; peptide-G ["glasstide", a PKG substrate (Glass & Krebs, 1979)]; peptide-H [a histone H1 fragment

[2] C_{APL-A1} and C_{APL-A2} bind the PKIP 20-mer (TTYADFIASGRTGRRNAIHD) equally well (data not shown).

[3] Olsen and Uhler (1989) obtained surprisingly low values of k_{cat} for mouse Cα and Cβ purified using their affinity column. This problem was not apparent in the cases of C_{APL-A1} and C_{APL-A2}.

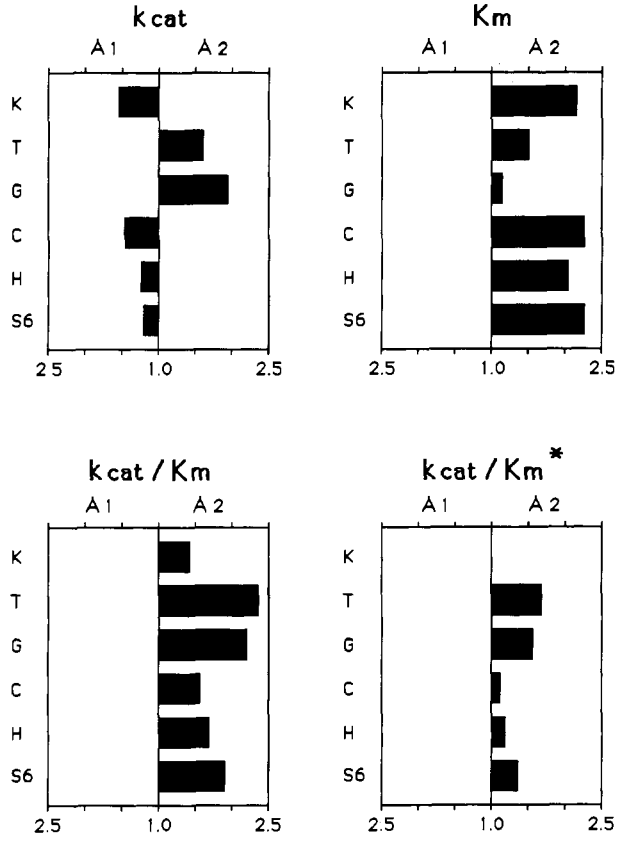

FIGURE 4: Graphical comparison of kinetic constants determined for C_{APL-A1} and C_{APL-A2} with a variety of peptide substrates. The ratios of the kinetic constants determined for C_{APL-A1} and C_{APL-A2} (Table II) are compared graphically. Where C_{APL-A1} prefers a substrate, according to a given parameter (higher k_{cat}, lower K_m, higher k_{cat}/K_m), the bar showing the ratio is to the left; where C_{APL-A2} prefers a substrate, the bar is to the right. In the panel at bottom right $(k_{cat}/K_m)^*$ is the ratio of $(k_{cat}/K_m)_{peptide-X}/(k_{cat}/K_m)_{kemptide}$ values for C_{APL-A1} and C_{APL-A2}, which is a measure of the extent to which the relative substrate specificity of C_{APL-A1} and C_{APL-A2} for a given peptide differs from the relative substrate specificity of C_{APL-A1} and C_{APL-A2} for kemptide. The peptides are described in the text and in Table II.

(Pomeranz et al., 1977)]; peptide-C [a protein kinase C substrate (House & Kemp, 1987)]; and peptide-S6 [an S6 kinase substrate (Pelech et al., 1986)]. In Figure 4, the ratios of the constants for C_{APL-A1} and C_{APL-A2} are compared graphically. Where C_{APL-A1} prefers a substrate, according to a given parameter (higher k_{cat}, lower K_m, higher k_{cat}/K_m), the bar showing the ratio is to the left; where C_{APL-A2} prefers a substrate, the bar is to the right. In terms of k_{cat}, peptide-K, -C, -H, and -S6 are preferred by C_{APL-A1}, while peptide-T and -G are preferred by C_{APL-A2}. In terms of K_m, all substrates are preferred by C_{APL-A2}, although the difference is not significant for peptide-G. Considering k_{cat}/K_m, all substrates show a modest preference for C_{APL-A2}: the ratio $(k_{cat-A1}/K_{m-A1})/(k_{cat-A2}/K_{m-A2})$ ranges from 0.43 for peptide-T to 0.71 for kemptide itself. This means, for example, that given a mixture of peptide-K and -T, C_{APL-A2} would phosphorylate 1.7 times more peptide-T relative to kemptide than would C_{APL-A1}. This aspect of substrate specificity is embodied in the parameter $(k_{cat}/K_m)^*_{-A1}/(k_{cat}/K_m)^*_{-A2}$ shown for all the peptides examined in Figure 4 (bottom right panel).

RIIα Is Phosphorylated by C_{APL-A1} More Rapidly Than by C_{APL-A2}. The rates of phosphorylation of RIIα by C_{APL-A1} and C_{APL-A2} were measured at very low ATP concentrations, where the rates depend on nucleotide concentration. As the K_m values for ATP for C_{APL-A1} and C_{APL-A2} are very similar (see below), the relative rates of 2.7:1 (C_{APL-A1}:C_{APL-A2}, data not shown) probably reflect k_{cat-A1}/k_{cat-A2} for RIIα, although this cannot be stated with certainty because details of the enzyme mechanism remain unclear (Edelman et al., 1987). It is presumed that the *Aplysia* C subunits phosphorylate RIIα at the sequence RRVSVC, which is the site in the inhibitory domain phosphorylated by the bovine C subunit (Taylor et al., 1990). cAMP was added to avoid difficulties with traces of residual cyclic nucleotide that might have differed in concentration between the two C subunit preparations.

Although this is one of the largest differences between substrates that we have noted for C_{APL-A1} and C_{APL-A2}, it may have little physiological significance as no RII isoform has yet been clearly identified in an invertebrate (Kalderon & Rubin, 1988; Lu et al., 1990; Bergold et al., 1991). However, it does suggest that there may be yet larger differences between protein substrates than those we have noted with peptides, perhaps reflecting the spatial organization of the 10 residues that differ between the two forms of *Aplysia* C subunit.

ATP Is a Specific Substrate for C_{APL-A1} and C_{APL-A2} and Exhibits a Similar K_m for Each Subunit. Using 200 μM kemptide as substrate, the K_m values for ATP for the two isoforms were very similar: 33 ± 1 μM for C_{APL-A1} and 30 ± 1 μM for C_{APL-A2}. This is not surprising as the differences in the amino acid sequences of C_{APL-A1} and C_{APL-A2} are in a region of the polypeptide chain that is primarily responsible for peptide substrate rather than ATP binding (Taylor et al., 1990). Of course, both subunits would be saturated with nucleotide at intracellular Mg^{2+} and ATP concentrations even if the K_m values differed by an order of magnitude. Phosphorylation of kemptide by 10 μM [γ-^{32}P]ATP was not inhibited (≤5%) by unlabeled 1 mM rGTP, rUTP, or rCTP. Unlabeled 1 mM rATP, dATP, or ddATP all prevented phosphorylation (≥90%) under the same conditions, suggesting that both C_{APL-A1} and C_{APL-A2} exhibit a strong preference for adenosine, as is the case for the mammalian subunits, but not all Ser/Thr protein kinases [casein kinase II and cdc2 protein kinase both utilize ATP and GTP (Maller, 1990)].

RIα Binds C_{APL-A2} More Tightly Than C_{APL-A1}. Many factors can complicate the measurement of a true K_d for R when substrate phosphorylation is used as an assay for free C subunit. For example, binding of R is competitive with substrate but dissociation of the R·C complex occurs on the same time scale as kemptide phosphorylation (Buxbaum & Dudai, 1989). Therefore, like others (Kuret et al., 1988), we decided to measure the IC_{50} of R under carefully controlled conditions. The IC_{50} will depend, among other things, on variables such as the C subunit concentration, the nature of the substrate, the length of the assay and the order of addition of assay components, all of which were fixed in our experiments. C_{APL-A2} was inhibited by concentrations of rRIα 3.5 ± 0.1 times lower than those required to inhibit C_{APL-A1} (Figure 5). The IC_{50} for C_{APL-A1} was 110 pM in the presence of ATP (Neitzel et al., 1991). Because C_{APL-A2} binds kemptide more tightly than C_{APL-A1} and must significantly deplete free R in the assay, this ratio of IC_{50} values is probably lower than the ratio of the dissociation constants.

Discussion

Functional Significance of the Exon Duplication in the C_{APL-A} Gene. The amino acid sequences encoded by exon cassettes A1 and A2 differ as much from each other as they do from the corresponding mammalian sequences (Figure 1). This suggests that the duplication that created the exons has been retained from an ancient time of origin to the present and presumably serves a useful function. An organism that

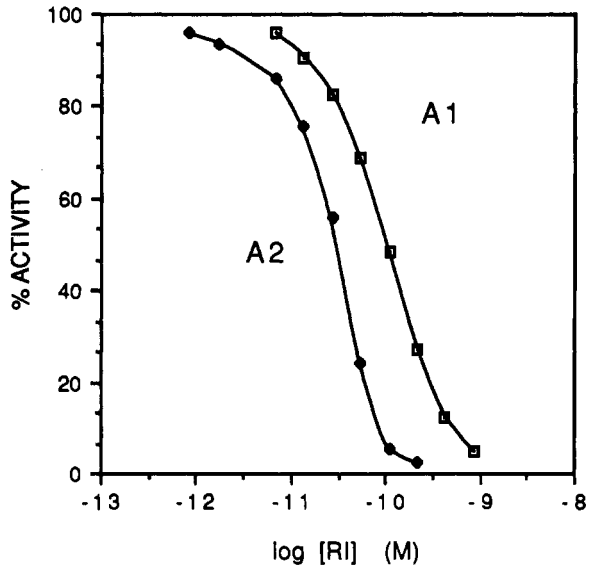

FIGURE 5: Bovine RIα inhibits C_{APL-A2} more strongly than C_{APL-A1}. C_{APL-A1} and C_{APL-A2} were titrated with recombinant bovine RIα under identical conditions as described in the text. Residual kinase activity was assayed with 100 μM kemptide and is plotted as a percentage of the value obtained with kemptide in the absence of R subunit. The experiment was carried out three times in duplicate, each time with a different preparation of cAMP-free rRIα. The data shown are from one representative experiment.

has duplicated an exon with a specific role, rather than an entire gene, might have a selective advantage as this would facilitate a natural form of segment-directed mutagenesis. On the other hand, it should be noted that duplicated exons such as A1 and A2 might survive in the genome for a comparatively long period, as dispensible if not redundant DNA, because they are relatively small targets for inactivation.

As the *Aplysia* C subunit isoforms have distinguishable but similar properties, it is unlikely that they have highly specific functions. It is more likely that combinations of kinase gene products with overlapping activities are involved in fine-tuning the properties of cells. The combined kinetic properties of closely related enzymes, such as C_{APL-A1} and C_{APL-A2}, may produce activities that cannot be generated with a single enzyme isoform, for example, a multiphasic temporal response upon activation, a broadening of substrate specificity, a wider dynamic range of response to regulatory effectors (cAMP in the present case), and greater versatility in subcellular localization and translocation. In the *Aplysia* nervous system transcripts containing A1 are ~3 times more prevalent than those containing A2, while in the ovotestis A1 transcripts are ~5 times less prevalent than those containing A2 (Beushausen et al., 1988; Beushausen et al., in preparation). Differential expression may therefore contribute to subtle differences in cAMP-mediated intracellular signaling. It remains possible that a change in the ratio of the closely related isoforms contributes to neuronal plasticity.

Diverse Catalytic and Regulatory Subunits of PKA and Related Kinases Are Generated by a Variety of Mechanisms in Other Species. The Ser/Thr protein kinases regulated by the second messengers cAMP, cGMP, and Ca^{2+}/diacylglycerol (the AGC kinases) are closely related (Hanks et al., 1988). Numerous isoforms of each of these kinases are being discovered, particularly in the central nervous system (Cadd & McKnight, 1989; Kikkawa et al., 1989). For example, three genes encoding C subunits of PKA have been identified in mammals [Cα, Cβ, and Cγ (Chrivia et al., 1988; Beebe et al., 1990)], and alternatively spliced transcripts of the Cβ gene encode polypeptides with two different N-termini (Wiemann et al., 1991). In *Drosophila* the single C subunit gene identified so far has no introns in the coding region (Foster et al., 1988; Kalderon & Rubin, 1988), while in *Caenorhabditis elegans* alternative splicing affects the C-terminus of the coding region (Gross et al., 1990). Multiple regulatory subunits of PKA have also been found in several species, including mammals (Clegg et al., 1988), *Drosophila* (Kalderon & Rubin, 1988), and *Aplysia* (Bergold et al., 1991). Isoforms arising from multiple genes and alternative splicing also exist for PKG (Francis et al., 1989; Kalderon & Rubin, 1989; Wernet et al., 1989) and for PKC (Ono et al., 1988; Ohno et al., 1988; Kikkawa et al., 1989; Akita et al., 1990). Because the holoenzyme of PKA consists of two R and two C subunits, a great variety of holoenzymes can be generated by combinatorial expression. For example, in *Aplysia* neurons, the four low molecular weight R subunits (Eppler et al., 1986; Bergold et al., 1991) and four C subunits [Beushausen et al. (1988) and manuscript in preparation] could yield 136 holoenzymes.

Substrate Specificity of Other AGC Kinases and Their Isoforms. The substrate specificity of the AGC kinases has been best quantitated in vitro with synthetic substrates. For example, when PKA and PKG were compared with peptides chosen as selective substrates, PKA was found to bind kemptide more tightly (PKA, K_m 16 μM, k_{cat} 14 s^{-1}; PKG, K_m 180 μM, k_{cat} 32 s^{-1}), while PKG had greater affinity for peptide-G (PKA, K_m 110 μM, k_{cat} 0.8 s^{-1}; PKG, K_m 22 μM, k_{cat} 5.6 s^{-1}; Glass & Krebs, 1979). The differences between C_{APL-A1} and C_{APL-A2} should be placed in this context, i.e., PKA and PKG do exhibit measurable selectivity toward synthetic substrates, but they are certainly not as discriminating as most enzymes. Accordingly, the AGC kinases phosphorylate many common substrates in vivo (Glass & Krebs, 1980; Edelman et al., 1987; Kemp & Pearson, 1990). It has been especially difficult to differentiate PKA and PKG substrates of physiological significance, although progress is now being made in this area (Thomas et al., 1990), which suggests that physiological substrates for C_{APL-A1} and C_{APL-A2} may exist for which differences in specificity are more pronounced than those measured here with synthetic peptides.

In parallel with our work, others have measured the substrate kinetics of isoforms of various AGC kinases. Olsen and Uhler (1989) found no differences in K_m values for kemptide and ATP between mouse Cα and Cβ1, which are 95% identical in sequence in the kinase domain. The interactions of these isoforms with R subunits are also indistinguishable (Cadd et al., 1990). The substrate specificities of PKG Iα and Iβ are almost identical, which is perhaps unsurprising as they differ only in the N-terminal regulatory domain (Francis et al., 1989; Wernet et al., 1989). Differences of the same order of magnitude as those seen between C_{APL-A1} and C_{APL-A2} have been found for the α, βI, and γ isoforms of Ca^{2+}-dependent PKC (Marais & Parker, 1989), which are 75–85% identical in the kinase domain (Ono et al., 1988). Greater differences in substrate specificity exist between the Ca^{2+}-dependent and one of the Ca^{2+}-independent PKCs (PKCε; Schaap et al., 1990), which differ considerably in their catalytic domains [PKCε 60–65% identity to PKCα, β, γ (Ohno et al., 1988)]. These recent measurements reinforce the idea that the AGC kinases exhibit an almost continuous spectrum of catalytic specificity.

Overlapping Regulation of the AGC Kinases. The regulation of PKA and PKG can be complicated by "cross talk" between the two enzymes during activation. For example, in sea urchin spermatozoa, which apparently lack PKG, the rise in intracellular cGMP after stimulation with egg peptides

activates PKA (Bentley et al., 1987). The regulation of PKC is also complex. All forms require diacylglycerol and phospholipid, but only a subset require Ca^{2+} (Ohno et al., 1988; Akita et al., 1990; Schaap et al., 1990). The regulation of the AGC kinases is further complicated by the heterogeneity of regulatory domains and subunits. For example, PKA holoenzyme containing RIβ, in combination with either Cα or Cβ, is activated at concentrations of cAMP 3–7 times lower than holoenzyme containing RIα (Cadd et al., 1990). The inhibitory properties of the *Aplysia* neuronal R subunits remain to be explored, although it is already clear from the present work that the C subunits, $C_{APL\text{-}A1}$ and $C_{APL\text{-}A2}$, interact differently with R. Subtle differences in the regulation of PKG Iα and Iβ (Wolfe et al., 1989) and the Ca^{2+}-dependent PKCs [e.g., Marais and Parker (1989)] have also been noted.

Subcellular Distribution and Translocation of the AGC Kinases. As yet, there is no evidence for the differential subcellular localization of $C_{APL\text{-}A1}$ and $C_{APL\text{-}A2}$. Indeed, subcellular localization of PKA holoenzyme is thought to be determined by R subunits (Scott et al., 1990; Luo et al., 1990, and references therein). In *Aplysia* neurons, the N1 regulatory subunit is particulate and the N5 subunit soluble, while N2, 3, and 4 exhibit a more complex distribution (Eppler et al., 1986). It is possible then that differential association of R subunits with $C_{APL\text{-}A1}$ and $C_{APL\text{-}A2}$ results in differential subcellular localization of the two activities. Kinase subunits can relocate after holoenzyme activation (Meinkoth et al., 1990; Adams et al., 1991, and references therein), but no evidence is available concerning the translocation of $C_{APL\text{-}A1}$ and $C_{APL\text{-}A2}$. Differential subcellular localization and translocation of other AGC kinases, notably PKC [e.g., Hocevar and Fields (1991)], have also been observed.

Most Signal Transduction Proteins Exhibit a Spectrum of Activities Through the Existence of Isoforms. The known network of interactive signal-transduction pathways is highly complex even when protein isoforms are not considered [e.g., Rozengurt (1986) and Cantley et al. (1991)]. However, isoforms must be accounted for as polypeptides with overlapping properties are being found at all levels of signal transduction, including, besides protein kinases and phosphatases, receptors (Schofield et al., 1990), G proteins (Ross, 1989), ligand- and voltage-gated channels (Schofield et al., 1990; Jan & Jan, 1990), and transcription factors [e.g., Nakabeppu and Nathans (1991)]. In addition, the combinatorial expression of subunits to form heterooligomeric proteins, such as the holoenzymes of PKA (see above) and K channels (Christie et al., 1990), yields myriads of protein forms. This intricacy is further complicated by the complex spatial organization of signal-transduction molecules and by translocation events. The resulting maze of overlapping, interacting pathways must be considered, for example, by pharmacologists who seek to interfere selectively with cell signaling.

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